

PATENT COOPERATION TREATY

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From the
INTERNATIONAL PRELIMINARY EXAMINING

PCT

To:

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27 DEC 2000

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing (day/month/year)	19.12.2000
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Applicant's or agent's file reference PS/P21245WO	IMPORTANT NOTIFICATION
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International application No. PCT/EP99/07720	International filing date (day/month/year) 10/09/1999	Priority date (day/month/year) 10/09/1998
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Applicant ERASMUS UNIVERSITEIT ROTTERDAM et al.
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1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PS/P21245WO	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP99/07720	International filing date (day/month/year) 10/09/1999	Priority date (day/month/year) 10/09/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant ERASMUS UNIVERSITEIT ROTTERDAM et al.		



1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

 These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 04/04/2000	Date of completion of this report 19.12.2000
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Maucher, C Telephone No. +49 89 2399 7415 

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP99/07720

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:

Description, pages:

1,3-24	as originally filed	
2	with telefax of	20/11/2000

Claims, No.:

1-22	with telefax of	20/11/2000
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Drawings, sheets:

1/1	as originally filed
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2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP99/07720

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
☒ claims Nos. 1-8, 11-20 with respect to industrial applicability.

because:

- ☒ the said international application, or the said claims Nos. 1-8, 11-20 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

- ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
☐ the computer readable form has not been furnished or does not comply with the standard.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability;
citations and explanations supporting such statement**

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP99/07720

1. Statement

Novelty (N)	Yes:	Claims	1-22
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-22
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	9-10, 21-22
	No:	Claims	

2. Citations and explanations **see separate sheet**

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP99/07720

The arguments filed by the applicant with a telefax of 20.11.2000 have been considered for the establishment of this report.

Point I:

1. Newly filed description page 2 and newly filed claims 1-22 (with a telefax of 20.11.2000) meet the requirements of Article 34(2)(b) PCT.
2. This written opinion also takes into consideration pages 1-2 of the Sequence listing (i.e. information concerning SEQ ID NO's 1-2) filed with a letter of 21.12.99.

Point III:

Claims 1-8 and 11-20 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Point V:

Reference is made to the following documents:

- D1: JCE&M, vol. 82, no. 6, 1997, p 1772-1775
- D2: WO-A-97 40187
- D3: BRITISH JOURNAL OF NUTRITION, vol. 79, 1998, p 315-327
- D4: JCE&M, vol. 82, no. 6, 1997, pages 1772-1775, JCE&M, vol. 83, no. 7, July 1998, p 2255-2259
- D5: NATURE MEDICINE, vol. 1, no. 12, 1995, p 1309-1310

1. Novelty and Inventive Step

- 1.1. The subject-matter of **claims 1-16 is novel** (Article 33(2) PCT) in view of the

available prior art D1, D4 or D5 in that it discloses a method of "determining susceptibility to heart disease" by determining which of the B/b, A/a or T/t alleles of the BsmI, ApaI or TaqI sites of the vitamin D receptor is/are present, wherein the b, a, or T alleles are associated with risk of heart disease (see p 4-5, bridging sentence).

D1, D4 and D5 disclose that the vitamin D receptor (VDR) polymorphisms (D1, p 1772, last par, l 3, 10, 12-13, p 1774, last par, sentences 1 and 3; D4, p 2255, par of DNA analysis; D5, p 1310-1311, bridging par) are associated with hyperparathyroidism (HPT) (D1, D4: title, D5: abstract). D3 (p 317, last sentence to p 319, 1st full sentence) describes hypertension as a feature of HPT.

However, none of the prior art documents describes a direct correlation of VDR polymorphisms and heart disease. Furthermore, not all patients having HPT suffer from hypertension and not all patients with hypertension develop myocardial infarction.

Therefore, there is no direct link in subjects between HPT and risk of myocardial infarction. Consequently, the method disclosed in claims 1-16, wherein the link from heart disease to VDR polymorphisms is established, was not obvious in the light of the available prior art and does meet the requirements of Article 33(3) PCT.

- 1.2. The subject-matter of **claims 17-19** is **novel** in view of the available prior art. It is distinguished therefrom in that a method is claimed for "predicting the response of a subject to treatment" by analysing which of the B/b, A/a or T/t allele/s of the vitamin D receptor is/are present.

No prior art document, either if taken alone or in any combination, teaches or suggests the correlation of heart disease and the determination of whether the B/b, A/a or T/t allele/s of the vitamin D receptor is/are present for predicting the response of a subject to treatment. Therefore, the subject-matter of **claims 17-19** **meets the requirements of Article 33(3) PCT.**

- 1.3. The subject-matter of **claims 20-22** is **novel and inventive** in the light of the closest prior art, since no prior art document, either if taken alone or in any

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combination, discloses the correlation of vitamin D receptor alleles with risk of heart disease. Therefore, provided that a reference to the specific alleles which were found to be linked to risk of heart disease is mentioned in claims 20-22, an inventive step can be acknowledged (see also VIII, 1, 2).

2. Industrial Applicability

For the assessment of the present claims 1-8 and 11-20 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Point VI:

The priority of the present application is valid. Thus, the intermediate document "THE FASEB JOURNAL, vol. 12, October 1998, pages 1409-1417" published between the priority date and the filing date is not considered to be state of the art and therefore not taken into account for the examination of the current application.

Point VII:

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the document D2 is not mentioned in the description, nor is this document identified therein.

Point VIII:

1. Claims 20-22 do not meet the requirements of Article 6 PCT, in that the matter for which protection is sought is not clearly defined. The claims attempt to define the

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

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components of the diagnostic kit of interest in terms of the result to be achieved which merely amounts to a statement of the underlying problem. The technical features necessary for achieving this result have not been added.

2. Claims 20-22 are silent about the determination of the susceptibility to heart disease: it is not derivable from the claims which allele(s) is/are connected with heart disease susceptibility (see description p 3, l 16-18). Thus, said claims do not meet the requirements of Article 6 PCT, because the novel and inventive feature, being the specific alleles, is missing.

mediates hormone response by initiating gene transcription and mRNA translation⁶. Involvement of the vitamin D endocrine system in the etiology of heart disease was suggested by epidemiological studies which found serum levels of 25-hydroxyvitamin D₃ to be reduced in myocardial infarction patients compared with control individuals⁷. Furthermore, congestive heart failure associated with vitamin D deficiency has been described in a three and a half month old child⁸. The involvement of the vitamin D endocrine system in cardiac arrhythmias was suggested by case reports involving the observation of atrial flutter in a foetus with X-linked vitamin D resistant rickets⁹ and sick sinus syndrome that was cured by administration of vitamin D to a 77-year old caucasian woman¹⁰. Further studies in the rat have also demonstrated an important role of the vitamin D endocrine system in cardiovascular function, wherein vitamin D₃-deficiency can lead to large increases in the contractile function of the heart¹⁷⁻²⁰.

The above studies establish a link between the vitamin D endocrine system and risk of heart disease in an individual. However, the genetic component of heart disease such as myocardial infarction or cardiac arrhythmia is poorly defined. As a result, methods of diagnosing risk of heart disease, or treating those suffering from heart disease, are restricted.

Thus, in a first aspect of the present invention, there is provided a method of determining susceptibility to heart disease in a subject, said method comprising analysing genetic material of a subject to determine which allele(s) of the vitamin D receptor is/are present.

The vitamin D receptor gene (12q12) comprises inherited polymorphisms between exon 7 and the 3' UTR of the VDR gene, as shown in Figure 1. These alleles are

CLAIMS

1. A method of determining susceptibility to heart disease in a subject, said method comprising analysing genetic material of a subject to determine which
5 allele(s) of the vitamin D receptor gene is/are present.
2. A method of determining susceptibility to heart disease according to claim 1, said method comprising analysing the genetic material of a subject to determine which
10 of the B/b, A/a or T/t alleles of the *BsmI*, *ApaI* or *TaqI* sites of the vitamin D receptor are present.
3. A method of determining susceptibility to heart disease according to claim 1, said method comprising analysing the genetic material of a subject to determine the
15 haplotype of the *BsmI*, *ApaI* or *TaqI* alleles of the vitamin D receptor.
4. A method according to claim 3 wherein the haplotype may be determined by amplification of a relevant portion of the vitamin D receptor gene, followed by restriction enzyme digestion.
- 20 5. A method of determining susceptibility to heart disease according to claims 1 to 4, said method comprising determining the copy number of the B/b, A/a or T/t alleles or haplotype of the vitamin D receptor.
6. A method according to any one of the preceding claims further comprising
25 determining whether the allele(s) present is/are associated with risk of heart disease.
7. A method according to claim 6 comprising comparing the allele(s) present in the genetic material of the subject with those known to be associated allele(s) of vitamin D receptor genotypes having known degrees of risk of heart disease

8. A method according to any one of the previous claims wherein said method further comprises determining aspects of calcium metabolism in a subject.
- 5 9. A method according to claim 8, wherein daily calcium intake of a subject is measured.
- 10 10. A method according to any one of the preceding claims, wherein said method is performed *in vitro*.
11. A method according to claim 10 wherein said method is performed on blood or tissue samples of a subject.
- 15 12. A method according to any one of the preceding claims wherein the subject is a mammal.
13. A method according to claim 12 wherein the subject is a human.
- 20 14. A method according to claims 12 or 13 wherein the subject is male.
15. A method according to any one of the previous claims for determining susceptibility of a subject to atrial or ventricular hypertrophy, aortic calcification, arrhythmia, myocardial infarction, or hypertension.
- 25 16. A method according to any one of the preceding claims further comprising treating the subject to reduce the risk of heart disease.
- 30 17. A method according to claim 16 wherein suitable treatments may include modifications to lifestyle, regular exercise, changes in diet or pharmaceutical preparations.

18. A method of predicting the response of a subject to treatment, said method comprising analysing genetic material of a subject to determine which allele(s) of the vitamin D receptor gene is/are present.

5 19. A method according to claim 18 wherein said subject is first diagnosed as being susceptible to heart disease in accordance with any one of claims 1 to 17.

20. A method according to claims 18 or 19 further comprising administering the appropriate treatment.

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21. Use of a kit to determine susceptibility to heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene, and (ii) means for determining which allele(s) of said gene is/are present.

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22. A kit for determining susceptibility to heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene; (ii) means for determining which allele(s) of said gene is/are present; and (iii) means for indicating correlation between said

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allele(s) and risk of heart disease.

23. A kit according to claim 22, said kit comprising DNA control samples, for comparison with DNA sequences of a subject.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/68	A1	(11) International Publication Number: WO 00/15840 (43) International Publication Date: 23 March 2000 (23.03.00)
(21) International Application Number: PCT/EP99/07720 (22) International Filing Date: 10 September 1999 (10.09.99) (30) Priority Data: 9819764.3 10 September 1998 (10.09.98) GB (71) Applicant (for all designated States except US): ERASMUS UNIVERSITEIT ROTTERDAM [NL/NL]; Burgemeester Oudlaan 50, NL-3062 PA Rotterdam (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): UITTERLINDEN, An- dreas, Gerardus [NL/NL]; Limes 67, NL-3176 TE Poor- tugaal (NL). VAN LEEUWEN, Petrus, Thomas, Maria [NL/NL]; Maluslaan 23, NL-1185 KZ Amstelveen (NL). POLS, HuiBERT, Adriaan, Pieter [NL/NL]; Campanulhof 21, NL-3355 BH Papendrecht (NL). (74) Agents: SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: METHOD FOR DETERMINING SUSCEPTIBILITY TO HEART DISEASE BY SCREENING POLYMORPHISMS IN THE VITAMIN D RECEPTOR GENE		
(57) Abstract The present invention relates to prognostic method and means for determining susceptibility to heart disease in a subject by screening for polymorphisms in the Vitamin D receptor gene. In particular, the present invention provides a method for determining susceptibility to heart disease, the method comprising analysing the genetic material of a subject to determine which of the B/b, A/a or T/t alleles of the restriction enzyme sites <i>BsmI</i> , <i>Apal</i> and <i>TaqI</i> respectively are present. Specific combinations of alleles represent a haplotype which is associated with susceptibility to heart disease.		

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METHOD FOR DETERMINING SUSCEPTIBILITY TO HEART DISEASE BY SCREENING POLYMORPHISMS IN THE VITAMIN D RECEPTOR GENE

The present invention relates to a prognostic method and prognostic means based on polymorphisms in the vitamin D receptor gene. In particular, the present invention
5 relates to a method for determining susceptibility to heart disease by screening for polymorphisms in the vitamin D receptor gene.

Heart disease such as myocardial infarction is a complex phenotype caused by interaction of a number of genetic and environmental factors.¹ Advances in
10 molecular genetics have led to the identification of a number of genetic risk factors for heart disease, such as gene variants involved in thrombosis^{2,3} and lipid metabolism⁴. However, another important risk factor for heart disease such as myocardial infarction is a deregulated calcium homeostasis which is required for
15 maintenance of neuromuscular activity⁵. Disturbances of the calcium balance have been implicated in hypertension, ventricular hypertrophy, aortic calcification and arrhythmias all of which are important risk factors associated with myocardial infarction.

Vitamin D is a potent regulator of bone and calcium homeostasis, as well as of
20 cellular differentiation and replication in many tissues, and mediates its effects through the vitamin D receptor (VDR). Cloning of the vitamin D receptor has shown it to be a member of the ligand-activated superfamily, which are natural regulators of a number of physiological and developmental processes. The major physiological function of the active metabolite of vitamin D, i.e. 1,25(OH)₂D₃, is to
25 maintain serum calcium levels in the normal range. This is accomplished by stimulating the small intestine to increase its efficiency of absorbing calcium from the diet and to mobilize calcium stores from bone. The action of the hormone is mediated by the vitamin D receptor (VDR), a steroid transcription factor that

mediates hormone response by initiating gene transcription and mRNA translation⁶. Involvement of the vitamin D endocrine system in the etiology of heart disease was suggested by epidemiological studies which found serum levels of 25-hydroxyvitamin D₃ to be reduced in myocardial infarction patients compared with control individuals⁷. Furthermore, congestive heart failure associated with vitamin D deficiency has been described in a three and a half month old child⁸. The involvement of the vitamin D endocrine system in cardiac arrhythmias was suggested by case reports involving the observation of atrial flutter in a foetus with X-linked vitamin D resistant rickets⁹ and sick sinus syndrome that was cured by administration of vitamin D to a 77-year old caucasian woman¹⁰. Further studies in the rat have also demonstrated an important role of the vitamin D endocrine system in cardiovascular function, wherein vitamin D₃-deficiency can lead to large increases in the contractile function of the heart¹⁷⁻²⁰

The above studies establish a link between the vitamin D endocrine system and risk of heart disease in an individual. However, the genetic component of heart disease such as myocardial infarction or cardiac arrhythmia is poorly defined. As a result, methods of diagnosing risk of heart disease, or treating those suffering from heart disease, are restricted.

Thus, in a first aspect of the present invention, there is provided a method of determining susceptibility to heart disease in a subject, said method comprising analysing genetic material of a subject to determine which allele(s) of the vitamin D receptor is/are present.

The vitamin D receptor gene (12q12) comprises inherited polymorphisms between exon 7 and the 3' UTR of the VDR gene, as shown in Figure 1. These alleles are

denoted B/b, A/a and T/t for restriction enzyme sites *BsmI*, *ApaI* and *TaqI* respectively (or enzymatic or chemical procedures with similar specificities), where a lower case letter denotes the presence of a wild type restriction site which is capable of being cleaved, and a capital letter denotes the presence of a mutant restriction enzyme site which is not capable of being cleaved by the relevant restriction enzyme. For the purposes of the present invention, determination of which alleles are present in a particular gene may be referred to as determining the genotype of a subject for a particular gene. It is apparent from the above that each copy of the vitamin D receptor gene will comprise a specific combination of the three alleles, this combination being referred to as the haplotype of the gene. For example, the haplotype may be baT, indicating the presence of cleavable *BsmI* and *ApaI* sites, and a non-cleavable *TaqI* site. Direct haplotyping of the VDR gene has allowed five different haplotypes to be determined, of which three are common.¹⁶

The present invention is based on the discovery of a genetic component of heart disease. It has now been shown that presence of the b, a or T alleles, and in particular the baT haplotype of the vitamin D receptor is/are associated with increased risk of heart disease. Thus, the presence of such alleles of the vitamin D receptor gene may be used to determine susceptibility to heart disease.

Preferably, the method of the first aspect of the present invention comprises the additional step of determining whether the alleles present are associated with risk of heart disease. This may be performed by comparing the alleles present in the genetic material of the subject with genotypes of the vitamin D receptor having known degrees of risk of heart disease. For example, a visual aid detailing alleles and their relative risk of heart disease may be used to determine whether the genotype of the subject is associated with a high or low risk of heart disease.

The first aspect of the present invention may also comprise the additional step of determining aspects of calcium metabolism, such as calcium levels, in a subject. Preferably, the daily calcium intake is measured. This feature of the first aspect is based on the observation that the correlation between vitamin D receptor genotype and heart disease may be dependent upon dietary calcium intake.

The method of the first aspect may be performed *in vitro*. Preferably, the method is performed on tissue or fluid removed from the body of the subject. Thus, the present invention relates to a non-invasive method, the results of which provide an indication of susceptibility to heart disease but do not lead to a diagnosis upon which an immediate medical decision regarding treatment has to be made.

The present invention may be performed on any subject for whom it is desirable to determine risk of heart disease. Preferably, the subject may be a mammal. Most preferably, the method of the first aspect is performed on humans, preferably male.

Heart disease may include atrial or ventricular hypertrophy, aortic calcification, arrhythmia, hypertension, and most importantly, myocardial infarction.

In a preferred feature of the first aspect of the present invention, there is provided a method of determining susceptibility to heart disease, said method comprising analysing genetic material of a subject to determine which of the B/b, A/a or T/t alleles of the vitamin D receptor gene is/are present. The method may comprise determining whether one or more of the above alleles is present. The subject may further be classified as being heterozygous or homozygous for one or more alleles. Preferably, the method comprises the additional step of determining whether the allele(s) present is/are associated with risk of heart disease, wherein presence of the

b, a or T alleles is associated with increased risk of heart disease. Homozygosity for the a, b or T allele may further increase the susceptibility to heart disease in a subject.

5 In a preferred feature of the first aspect, there is provided a method of determining susceptibility to heart disease in a subject, said method comprising analysing the genetic material of a subject to determine the haplotype of the *BsmI*, *ApaI* and *TaqI* alleles at the vitamin D receptor. Preferably, said method comprises determining whether the haplotype of the subject is associated with risk of heart disease, wherein
10 the haplotype baT is associated with high risk of heart disease. A subject homozygous for said haplotype may be at a higher risk of heart disease than those heterozygous for the haplotype.

In a preferred feature of the first aspect, there is provided a method of determining
15 susceptibility to heart disease, said method comprising the additional step of determining the copy number of the B/b, A/a or T/t alleles or haplotype of the vitamin D receptor, where an increase in copy number is associated with increased risk of heart disease.

20 The present invention may be performed using any suitable method known in the art. Preferably, a tissue or fluid sample is first removed from a subject. Examples of suitable samples include blood, mouth or cheek cells, and hair samples containing roots. Other suitable samples would be known to the person skilled in the art. The genetic material is then extracted from the sample for diagnosis, using any suitable
25 method. The genetic material may be DNA or RNA, although preferably DNA is used. For example, the DNA may be extracted using the technique described in Sambrook *et al* (Molecular Cloning- A Laboratory Manual, Cold Spring Harbor Laboratory Press). Determination of the genotype of a subject may then be carried

out using the extracted DNA, employing any one of the following techniques:

- Southern blot analysis following digestion with one or more appropriate restriction enzymes.
- 5 ▪ PCR amplification followed by digestion with one or more appropriate restriction enzymes and, optionally, separation of digestion products by gel electrophoresis.
- Sequencing of a relevant gene fragment by any suitable method.
- Visualization of heteroduplex patterns, for example on PAA or agarose gels, where different patterns may indicate the presence of one or more specific alleles.
- 10 ▪ Separation of DNA fragments using denaturing gradient gels, wherein the degree of separation will depend upon the presence or absence of one or more polymorphic restriction sites.
- Separation using SSCP analysis, the patterns of which will depend upon the presence or absence of one or more polymorphic restriction sites.
- 15 ▪ Use of allele specific oligonucleotides, hybridization patterns of which will be specific for various combinations of alleles.
- Methods such as OLA, Taqman or dot-blot for the detection of known mutations.
- Visualization of DNA sites using fluorescent labelled probes for alleles of interest.
- 20 ▪ RFLP analysis

Where it is desirable to use particular restriction enzymes in performing the present invention, the skilled person will understand that enzymatic or chemical procedures having similar specificities may also be used. For example, restriction enzymes having similar specificity (isoschizomers) to those described herein may be used, or chemical degradation procedures with DNA or RNA cutting specificity.

25

Other techniques suitable for determining the genotype of a subject may be used in the present invention.

Where the haplotype of a gene is to be determined, it is preferable to use a direct
5 haplotyping method, as described in Uitterlinden *et al*¹⁶. In such a method, the
relevant portion of the gene is amplified and then subjected to restriction enzyme
digestion, in order to determine the presence or absence of restriction enzyme sites.
Thus, for example, where the haplotype of the vitamin D receptor gene is to be
determined, the portion of the gene between exon 7 and the 3' UTR may be
10 amplified, and the amplified DNA digested with the *BsmI*, *ApaI* or *TaqI* restriction
enzymes. Gel analysis may then be used to determine which alleles are present.

Preferably, a fragment may be amplified using polymerase chain reaction (PCR)
techniques, to produce copies which, where the fragment is of the vitamin D
15 receptor, are at least 1000 base pairs in length, and most preferably at least 1800
base pairs in length. PCR techniques are well known in the art, and it is within the
ambit of the skilled person to identify primers for amplification of the appropriate
region of the above genes, namely the region from exon 7 to the 3' UTR of the
vitamin D receptor gene. PCR techniques are described in EP-A-0200362 and EP-
20 A-0201184.

In a preferred feature of the first aspect, there is provided a method of determining
susceptibility to heart disease in a subject, said method comprising amplifying a
fragment comprising a portion of the region from exon 7 to the 3' UTR of the
25 vitamin D receptor gene, and determining which allele(s) in the vitamin D receptor
is/are present. Primers suitable for amplification of said portion of the vitamin D
receptor gene would be readily available to a person skilled in the art. Examples of
such primers include:

1. 5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' and/or
2. 5'-GCAACTCCTCATGGCTGAGGTCTC-3'

5 Where the amplified portion of the gene is larger than the above defined portion of the vitamin D receptor gene containing the relevant alleles, it is preferable to avoid the inclusion of gene sequences which comprise any one of the *BsmI*, *ApaI* or *TaqI* restriction sites.

10 In a second aspect of the present invention, there is provided a method of therapy, said method comprising treating a subject diagnosed as being at risk of heart disease, to reduce the risk of heart disease. Preferably, the subject is diagnosed as being at risk of heart disease in accordance with the first aspect of the present invention.

15 Therapy may in the form of preventative or palliative care. Suitable treatments include modifications to lifestyle, regular exercise and changes in diet. Suitable treatments, including pharmaceutical preparations, would be known to physicians and persons skilled in the art. Examples include ACE inhibitors, beta-blockers, calcium or vitamin D preparations, magnesium sulphate, thrombolytics and analgesics.

20

In a third aspect of the present invention, there is provided a method of predicting the response of a subject to treatment, said method comprising analysing genetic material of a subject to determine which allele(s) of the vitamin D receptor gene is/are present. Preferably, the method includes first determining whether the subject is susceptible to heart disease. Where a subject has been determined as susceptible to heart disease, the method may further comprise administering the appropriate treatment. The present aspect of the invention is based on the observation that agents, such as calcium channel blockers, which are useful for treatment of a variety of cardiovascular diseases may also be associated with cardiovascular morbidity in some cases. The effect of an

25

agent may therefore depend on the underlying cause of the heart disease. For example, the presence of the b, a or T alleles of the vitamin D receptor may result in modification of calcium uptake, leading to impaired cardiovascular function. Thus, in such a case it would be preferable to avoid the use of calcium channel blocking agents.

5

In a fourth aspect of the present invention, there is provided use of a kit to determine which allele(s) of the vitamin D receptor gene is/are present, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene, and (ii) means for determining which allele(s) is/are present in said gene.

10

Preferably, the primer molecules are suitable for amplification of at least a portion of the region between exon 7 and the 3'UTR of the vitamin D receptor gene. Examples of suitable primers are described above.

15

Means for determining which allele(s) is/are present in the vitamin D receptor gene, may include any reagents or molecules necessary for use in any of the methods described above. For example, where PCR followed by DNA digestion is used, said means preferably include PCR reagents and one or more of the *BsmI*, *ApaI*, or *TaqI* restriction enzymes. Where the method employs Southern Blotting, heteroduplex visualization, or fluorescent labelling techniques for example, probes which bind to the appropriate regions of the vitamin D receptor gene may be included. Where necessary, such probes may be labelled to allow detection, for example by nick-translation, radio- or fluorescent-labelling, or random primer extension whereby the non-labelled nucleotides serve as a template for the synthesis of labelled molecules. Other methods of labelling probes are well known in the art.

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In a preferred feature of the fourth aspect of the present invention, there is provided use of a kit further comprising means for indicating correlation between the genotype of a subject and risk of heart disease. Said means may be in the form of a chart or

visual aid, which indicate that presence of the b, a or T alleles or baT haplotype of the vitamin D receptor is/are associated with heart disease such as cardiac arrhythmia or myocardial infarction.

5 In a fifth aspect of the present invention, there is provided a kit for determining risk of heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene; (ii) means for determining which allele(s) is/are present in said gene; and (iii) means for indicating correlation between the allele(s) and risk of heart disease.

10

In a preferred feature of the fifth aspect, the kit may also comprise DNA control samples, for comparison with DNA sequences of a subject. The control samples may comprise the sequence of one or more alleles of the vitamin D receptor gene, or may comprise the sequence of various haplotypes.

15

Preferred features of each aspect of the present invention are as for each other aspect, mutatis mutandis.

20

The present invention will now be described in detail with reference to the following examples and figures in which:

FIGURE 1 is a schematic presentation of the region between exon 7 and the 3' UTR of the vitamin D receptor gene.

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Example 1 - Relationship between alleles of the vitamin D receptor gene and myocardial infarction, cardiac arrhythmias and dietary calcium intake in the elderly

METHODS

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Study Subjects

The Rotterdam Study is a population-based cohort study of 7983 subjects aged 55 or more years, residing in the Ommoord district of the city of Rotterdam in the Netherlands. The study was designed to document the occurrence of disease in the elderly in relation to several potential determinants.¹⁵ A total of 10,275 persons, of whom 9161 (89 percent) were living independently, were invited to participate in the study in 1991. In the independently living subjects, the overall response rate was 77 percent for home interview and 71 percent for examination in a research centre, including measurement of anthropometric characteristics and blood sampling. The Rotterdam Study was approved by the Medical Ethics Committee of the Erasmus University Medical School and written informed consent was obtained from each subject.

The analysis of the association between *VDR* genotype, myocardial infarction and arrhythmias was performed in a subgroup of men and women participating in the study. Baseline measurements, collected from 1990-1993, included and electrocardiogram (ECG), history of cardiovascular disease, established cardiovascular risk factors, and use of medication. A digitally stored ECG was available for 5931 independently living subjects from the study, but 1453 of these were excluded on the basis of age (> 80 yrs), use of a walking aid, diabetes mellitus or use of diuretic, estrogen, thyroid hormone or cytostatic drug therapy. From the 4478 remaining subjects, we studied a random sample of 2000 men and women aged 55 to 80 years. ECG data or DNA samples were not available for 22 subjects, resulting in a final study group of 1978 subjects.

Measurements

Height and weight were measured at the initial examination in a standing position without shoes. Myocardial infarction, including so called silent myocardial infarctions, was confirmed by ECG analysis. For 1725 men and women (87%) data

was available on dietary intakes of calcium (mg/day) during the preceding year. These were assessed by food frequency questionnaire and adjusted for energy intake. Age at menopause and current cigarette smoking were assessed by questionnaire. Cardiac arrhythmias were diagnosed by ECG analysis and included the occurrence of sinus irregularities, atrial flutter, and atrial fibrillation and were recorded, confirmed and classified by a physician. For 976 subjects data on cardiac arrhythmias were available (49%). Of these subjects, data on dietary calcium intake were available for 899 subjects (92%).

10 Determination of VDR Genotypes

Genomic DNA was extracted from peripheral venous blood samples according to standard procedures and the anonymous polymorphisms were detected by PCR as previously described¹⁶. Three anonymous polymorphic restriction enzyme recognition sites at the 3' end of the VDR gene, i.e. for BsmI, ApaI, and TaqI, were assessed in relation to each other by a direct molecular haplotyping PCR procedure which we developed¹⁶. This allows us to determine ophase of the alleles at each of the RFLP loci and as a result three frequent haplotype alleles are discerned, encoded 1 (baT; frequency 48%), 2 (BAt; frequency 40%), 3 (bAT; frequency 10%) combining to six genotypes encoded 11, 12, 13, 22, 23, and 33. Detailed information on haplotype alleles and genotype frequencies in the Rotterdam Study can be found elsewhere¹⁶. The PCR reaction mixture of 25 microlitres contained 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dideoxynucleotidetriphosphates, 150 ng of each primer, and 0.2 unit of Super Taq polymerase (HT Biotechnology, Cambridge, UK). The reactions were performed in a DNA thermocycler (model 480, Perkin Elmer) with a cycling protocol of 94°C, 60°C, and 72°C for 1 min each, for 28 cycles. Ten microlitres of PCR product were simultaneously digested with 5 units BsmI, 5 units ApaI, and 5 units TaqI restriction enzyme and 2 microlitres of a 10x concentrated buffer

(containing 150 mM Tris-HCl, pH 7.5, 250 mM NaCl, and 35 mM MgCl₂) by incubating for 30 min at 37°C, followed by a 30 min incubation at 65°C. The digestion products were analysed on a 1.2 percent agarose gel run in 44.5 mM Tris, 44.5 mM boric acid, 1 mM Na₂EDTA) for 300 volt-hours. Separation patterns were documented by Polaroid photography under UV-illumination (302 nm). To confirm the accuracy of genotyping, repeat analysis was performed on 200 random samples. No discrepancies were found.

Statistical Analysis

Relevant clinical variables were compared between the genotype groups by analysis of covariance and multiple linear regression was used to adjust values for confounding factors such as age, anthropometric variables, smoking. For the comparisons we made reference, heterozygote and homozygote groups for each of the VDR alleles. For example, for VDR allele1 the groups comprised 22, 23, and 33 for the reference group, 12, and 13 for the heterozygote group, and 11 for the homozygote group. The Chi-squared test was used to test for genotype distribution in men and women with and without myocardial infarction and with and without cardiac arrhythmias. Odds ratios (with 95 percent confidence interval) were calculated by multivariate logistic regression analysis to estimate the relative risk of myocardial infarction and cardiac arrhythmias. A multivariate regression model was designed to test for the influence of dietary calcium intake on the relationship between VDR genotype and myocardial infarction and VDR genotype with cardiac arrhythmias. For this analysis, subjects were categorised into groups of calcium intake defined by quartiles of the population. An interaction term was included in the regression analysis defined as gene allele dose multiplied with calcium-intake. P values for all statistical tests were two-sided.

RESULTS

Characteristics of the Study Subjects

Anthropometric, and dietary measurements in the men and women with the six VDR genotypes are shown in Table 1. The allele frequencies (1=48.6%; 2=40.6%; and 3=10.8%) and the distribution of genotypes were similar to that reported in a previous study²². There was no difference with regard to age, dietary calcium intake, smoking habits, body mass index, serum HDL-cholesterol, and serum cholesterol in the six genotype groups.

Association with Myocardial Infarction

The distribution of myocardial infarction by VDR genotype in men and women is shown in Table 2. There was modest over-representation of both men and women carrying the VDR haplotype allele 1 as compared with subjects without the allele although this did not reach significance. Logistic regression analysis showed that subjects in the heterozygous group had a 1.2 fold increased risk for myocardial infarction and the subjects in the homozygous group had a 1.5-fold increased risk, as compared with subjects not carrying the VDR allele 1. The gene dose effect was 1.2-fold increased risk per copy of the VDR allele 1. The gene dose effect was larger for women when compared to men (RRs were 1.1 (95% confidence interval 0.9 – 1.4) for men and 1.5 (95% confidence interval 1.1 – 2.2) for women) but the interaction term of sex times genotype in the regression model was not significant (P=0.65). The relative risks for myocardial infarction did not change after adjustment for potential confounding factors such as age, body mass index and smoking in the regression analysis. For VDR haplotype allele 2 and haplotype allele 3 no significant differences were observed (data not shown).

Modification of the Association by Dietary Calcium Intake

When the study subjects were stratified according to quartiles of dietary calcium intake, the differences in distribution of myocardial infarction by VDR genotype were restricted to individuals with the highest intake of calcium (1302 mg/day or more). These differences corresponded with 5.4-fold increased risk for heterozygote carriers and 8.3 -fold increased risk for homozygote carriers of VDR haplotype allele 1. Considering age, genotype and myocardial infarction together in a multivariate regression model, we found a significant modifying effect of dietary calcium intake on the VDR genotype effect on risk for myocardial infarction (P=0.04).

Association with Cardiac Arrhythmias

We subsequently analysed the distribution of cardiac arrhythmias by VDR genotype. We did not observe differences in the distribution of arrhythmias by VDR genotype in the total study group. However, in the group with high calcium intake (1302 mg/day or more) there was significant over-representation of cardiac arrhythmias for subjects carrying the VDR haplotype allele 1 as compared with subjects without the allele 1 (Table 4).

Logistic regression analysis showed that subjects in the heterozygous group had a 1.6 fold increased risk for cardiac arrhythmias and the subjects in the homozygous group had a 3.6-fold increased risk, as compared with subjects not carrying the VDR allele 1. The relative risks for cardiac arrhythmia did not change after adjustment for potential confounding factors such as age, body mass index and smoking in the regression analysis. Also when myocardial infarction was entered into the model the relative risks did not change.

DISCUSSION

Polymorphisms of the vitamin D receptor gene are associated with the risk of myocardial infarction. The increased risk was especially notable in subjects with a high dietary calcium intake. In this group, we also observed VDR haplotype 1 to be associated with increased risk of cardiac arrhythmias. Cardiac arrhythmias have been frequently observed following a myocardial infarct event. However, the VDR genotype dependent risk for cardiac arrhythmias we observe, is independent of myocardial infarction. This indicates that the genotype effect we observe for myocardial infarction is not mediated by the genotype effect on cardiac arrhythmias.

Our results indicate a genetic susceptibility of the risk for myocardial infarction and cardiac arrhythmias in subjects with a relatively high dietary calcium intake. This strongly suggest that the VDR genotype effect on myocardial infarction and cardiac arrhythmias is mediated by an effect on calcium transport. Treatment with calcium and vitamin D has been indicated for arrhythmias and hypertrophy and has been shown to improve myocardial function.

The hormone vitamin D has been shown to influence the Ca-flux in a variety of cell types including heart muscle cells. These rapid effects of the hormone are suggested to involve a non-genomic pathway by which the plasmalemmal VDR is coupled to plasma membrane Ca^{2+} channel proteins.

Calcium channel blocking agents are indicated for the treatment of a variety of cardiovascular diseases, including cardiac arrhythmias, but have also been found associated with increased cardiovascular morbidity^{21,22}. Therefore, the modification by calcium intake of the VDR genotype dependent effect on myocardial infarction we observe opens the possibility that genotyping at the VDR gene locus might be of value in predicting the response to treatment with some of these drugs. Prospective

clinical trials are needed to investigate the clinical and therapeutic implications of our results.

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CLAIMS

1. A method of determining susceptibility to heart disease in a subject, said method comprising analysing genetic material of a subject to determine which
5 allele(s) of the vitamin D receptor gene is/are present.
2. A method of determining susceptibility to heart disease according to claim 1, said method comprising analysing the genetic material of a subject to determine which
10 of the B/b, A/a or T/t alleles of the *BsmI*, *ApaI* or *TaqI* sites of the vitamin D receptor are present.
3. A method of determining susceptibility to heart disease according to claim 1, said method comprising analysing the genetic material of a subject to determine the
15 haplotype of the *BsmI*, *ApaI* or *TaqI* alleles of the vitamin D receptor.
4. A method according to claim 3 wherein the haplotype may be determined by amplification of a relevant portion of the vitamin D receptor gene, followed by restriction enzyme digestion.
- 20 5. A method of determining susceptibility to heart disease according to claims 1 to 4, said method comprising determining the copy number of the B/b, A/a or T/t alleles or haplotype of the vitamin D receptor.
6. A method according to any one of the preceding claims further comprising
25 determining whether the allele(s) present is/are associated with risk of heart disease.
7. A method according to claim 6 comprising comparing the allele(s) present in the genetic material of the subject with those known to be associated allele(s) of vitamin D receptor genotypes having known degrees of risk of heart disease

8. A method according to any one of the previous claims wherein said method further comprises determining aspects of calcium metabolism in a subject.

5 9. A method according to claim 8, wherein daily calcium intake of a subject is measured.

10. A method according to any one of the preceding claims, wherein said method is performed *in vitro*.

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11. A method according to claim 10 wherein said method is performed on blood or tissue samples of a subject.

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12. A method according to any one of the preceding claims wherein the subject is a mammal.

13. A method according to claim 12 wherein the subject is a human.

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14. A method according to claims 12 or 13 wherein the subject is male.

15. A method according to any one of the previous claims for determining susceptibility of a subject to atrial or ventricular hypertrophy, aortic calcification, arrhythmia, myocardial infarction, or hypertension.

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16. A method according to any one of the preceding claims further comprising treating the subject to reduce the risk of heart disease.

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17. A method according to claim 16 wherein suitable treatments may include modifications to lifestyle, regular exercise, changes in diet or pharmaceutical preparations.

18. A method of predicting the response of a subject to treatment, said method comprising analysing genetic material of a subject to determine which allele(s) of the vitamin D receptor gene is/are present.

5 19. A method according to claim 18 wherein said subject is first diagnosed as being susceptible to heart disease in accordance with any one of claims 1 to 17.

20. A method according to claims 18 or 19 further comprising administering the appropriate treatment.

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21. Use of a kit to determine susceptibility to heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene, and (ii) means for determining which allele(s) of said gene is/are present.

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22. A kit for determining susceptibility to heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene; (ii) means for determining which allele(s) of said gene is/are present; and (iii) means for indicating correlation between said

20 allele(s) and risk of heart disease.

23. A kit according to claim 22, said kit comprising DNA control samples, for comparison with DNA sequences of a subject.

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TABLE 1.
Characteristics of the Population According to VDR Genotype

CHARACTERISTIC†	VDR GENOTYPE					P-VALUE
	11	12	13	22	33	
Number (%)	493 (24.9)	735 (37.2)	202 (10.2)	351 (17.7)	170 (8.6)	27 (1.4)
Age (years)	67.0 ± 6.8	67.1 ± 6.8	67.2 ± 7.1	67.0 ± 7.1	66.9 ± 6.8	67.0 ± 7.1 §
Body Mass Index (kg/m ²)	26.1 ± 3.7	26.0 ± 3.3	25.8 ± 3.6	26.1 ± 3.4	26.0 ± 3.2	25.5 ± 2.9
Dietary calcium-intake (mg/day)	1116 ± 350	1122 ± 364	1122 ± 356	1092 ± 369	1094 ± 342	1158 ± 254
Serum HDL-cholesterol (mmol/l)	1.34 ± 0.37	1.35 ± 0.36	1.36 ± 0.34	1.35 ± 0.37	1.32 ± 0.33	1.36 ± 0.38
Serum cholesterol (mmol/l)	6.68 ± 1.21	6.63 ± 1.26	6.64 ± 1.16	6.60 ± 1.19	6.59 ± 1.21	6.60 ± 0.96
Current Smokers (%)	130 (26.4)	172 (23.4)	45 (22.3)	78 (22.2)	40 (23.5)	6 (22.2) ¶

† Values are means ± standard deviation; BMI is weight divided by the square height

§ P-value for ANOVA

¶ P-value for Chi-2 test

TABLE 2.

Myocardial Infarction According to *VDR allele 1* Genotype

	Men		Women		All	
	MI (%)	Total	MI (%)	Total	MI (%)	Total
Total	151 (15.8)	954	62 (6.1)	1024	213 (10.8)	1978
by <i>VDR allele 1</i> genotype						
Reference†	39 (14.7)	266	10 (3.5)	282	49 (8.9)	548
Heterozygotes	69 (15.4)	449	31 (6.4)	488	100 (10.7)	937
Homozygotes	43 (18.0)	239	21 (8.3)	254	64 (13.0)	493
χ^2	1.18		5.38		4.43	
P-VALUE	0.55		0.07		0.11	

Odds Ratios for Myocardial Infarct by *VDR allele 1* genotype [95% CI]

Crude

Reference	1.00	1.00	1.00
Heterozygotes	1.07 [0.72 - 1.71]	1.86 [0.90 - 3.85]	1.23 [0.86 - 1.76]
Homozygotes	1.28 [0.80 - 2.05]	2.48 [1.15 - 5.39]	1.53 [1.03 - 2.27]
per copy <i>VDR 1</i> allele	1.13 [0.89 - 1.44]	1.53 [1.07 - 2.20]	1.24 [1.02 - 1.51]

Age-, BMI-adjusted

Reference	1.00	1.00	1.00
Heterozygotes	1.11 [0.72 - 1.71]	1.77 [0.85 - 3.68]	1.22 [0.85 - 1.75]
Homozygotes	1.33 [0.82 - 2.14]	2.45 [1.12 - 5.34]	1.55 [1.04 - 2.30]
per copy <i>VDR 1</i> allele	1.15 [0.91 - 1.47]	1.53 [1.06 - 2.22]	1.25 [1.02 - 1.52]

† "Reference" includes *VDR* genotypes 22, 23, 33; "Heterozygotes" includes 12, 13;
"Homozygotes" includes 11

3 / 5

TABLE 3.

Myocardial Infarction According to VDR allele 1 Genotype by Quartiles of Dietary Calcium Intake

	< 877 mg/day		> 877, < 1076		> 1076, < 1302		≥ 1302	
	MI (%)	Total	MI (%)	Total	MI (%)	Total	MI (%)	Total
Total	43 (10.0)	432	50 (11.6)	431	45 (10.4)	432	49 (11.4)	430
by VDR allele 1 genotype								
Reference†	13 (9.9)	131	14 (12.5)	112	12 (9.6)	125	3 (2.6)	114
Heterozygotes	21 (10.5)	200	24 (11.8)	204	21 (10.0)	210	26 (12.6)	207
Homozygotes	9 (8.9)	101	12 (10.4)	115	12 (12.4)	97	20 (18.3)	109
χ^2	0.19		0.25		0.53		14.17	
P-VALUE	0.91		0.88		0.77		0.0008	
Odds Ratios for Myocardial Infarct by VDR allele 1 genotype [95% CI]								
Crude								
Reference	1.00		1.00		1.00		1.00	
Heterozygotes	1.09 [0.52 - 2.27]		0.93 [0.46 - 1.89]		1.04 [0.49 - 2.20]		5.40 [1.59 - 18.3]	
Homozygotes	0.90 [0.37 - 2.20]		0.82 [0.36 - 1.87]		1.32 [0.56 - 3.09]		8.31 [2.39 - 29.0]	

† "Reference" includes VDR genotypes 22, 23, 33; "Heterozygotes" includes 12, 13; "Homozygotes" includes 11

TABLE 4.
Myocardial Arrhythmias According to *VDR* allele 1 Genotype by Quartiles of Dietary Calcium Intake

	< 877 mg/day		> 877, < 1076		> 1076, < 1302		≥ 1302	
	MA (%)	Total	MA (%)	Total	MA (%)	Total	MA (%)	Total
Total	37 (12.1)	307	27 (9.2)	292	17 (5.6)	302	31 (10.1)	306
by <i>VDR</i> allele 1 genotype								
Reference†	16 (17.0)	94	6 (8.8)	68	7 (7.7)	91	5 (5.7)	88
Heterozygotes	14 (10.1)	138	14 (10.1)	138	6 (4.4)	135	12 (8.5)	141
Homozygotes	7 (9.3)	75	7 (8.1)	86	4 (5.3)	76	14 (18.2)	77
χ^2	3.19		0.27		1.11		7.80	
P-VALUE	0.20		0.87		0.58		0.02	
Odds Ratios for Myocardial arrhythmias by <i>VDR</i> allele 1 genotype [95% CI]								
Crude								
Reference	1.00		1.00		1.00		1.00	
Heterozygotes	0.57 [0.26 - 1.23]		1.13 [0.41 - 3.12]		0.54 [0.18 - 1.69]		1.60 [0.54 - 4.74]	
Homozygotes	0.51 [0.20 - 1.32]		0.92 [0.29 - 2.92]		0.69 [0.19 - 2.46]		3.63 [1.22 - 10.9]	

† "Reference" includes *VDR* genotypes 22, 23, 33; "Heterozygotes" includes 12, 13; "Homozygotes" includes 11

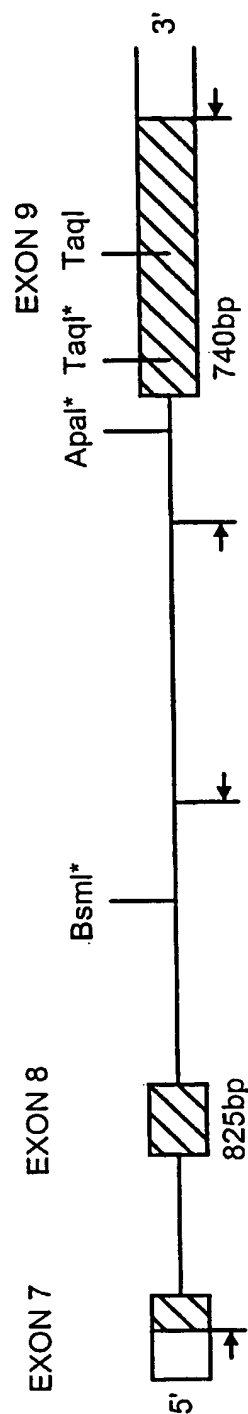


FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/07720

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CARLING ET AL.: "VITAMIN D RECEPTOR POLYMORPHISMS CORRELATE TO PARATHYROID CELL FUNCTION IN PRIMARY HYPERPARATHYROIDISM" JCE&M, vol. 82, no. 6, 1997, pages 1772-1775, XP000867630 the whole document	1-23
Y	CARLING ET AL.: "VITAMIN D RECEPTOR (VDR) AND PARATHYROID HORMONE MESSENGER RIBONUCLEIC ACID LEVELS CORRESPOND TO POLYMORPHIC VDR ALLELES IN HUMAN PARATHYROID TUMORS" JCE&M, vol. 83, no. 7, July 1998 (1998-07), pages 2255-2259, XP000876651 the whole document	1-23



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

11 February 2000

Date of mailing of the international search report

01/03/2000

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INTERNATIONAL SEARCH REPORT

Int. Jona: Application No

PCT/EP 99/07720

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CARLING ET AL.: "VITAMIN D RECEPTOR GENOTYPES IN PRIMARY HYPERPARATHYROIDISM" NATURE MEDICINE, vol. 1, no. 12, 1995, pages 1309-1310, XP002130405 cited in the application the whole document ---	1-23
Y	BOUCHER: "INADEQUATE VITAMIN D STATUS: DOES IT CONTRIBUTE TO THE DISORDERS COMPRISING SYNDROME "X"" BRITISH JOURNAL OF NUTRITION, vol. 79, 1998, pages 315-327, XP000870298 See pages 317, 319, "Evidence from studies in man" and page 321 "Vitamin D receptor, post receptor mechanisms" the whole document ---	1-23
Y	SCRAGG ET AL.: "MYOCARDIAL INFARCTION IS INVERSELY ASSOCIATED WITH PLASMA 25-HYDROXYVITAMIN D3 LEVELS: A COMMUNITY-BASED STUDY" INT.J. EPIDEMIOLOGY, vol. 19, no. 3, 1990, pages 559-563, XP000867323 cited in the application the whole document ---	1-23
Y	WO 97 40187 A (GEMINI INTERNATIONAL HOLDINGS ;SPECTOR TIMOTHY DAVID (GB); KEEN RI) 30 October 1997 (1997-10-30) the whole document ---	1-23
Y	WO 94 03633 A (GARVAN INST MED RES ;MORRISON NIGEL ALEXANDER (AU); EISMAN JOHN AL) 17 February 1994 (1994-02-17) the whole document ---	1-23
Y	UITTERLINDEN ET AL.: "A LARGE SCALE POPULATION BASED STUDY OF THE ASSOCIATION OF VITAMIN D RECEPTOR GENE POLYMORPHISMS WITH BONE MINERAL DENSITY" JOURNAL OF BONE AND MINERAL RESEARCH, vol. 11, no. 9, 1996, pages 1241-1248, XP000874270 cited in the application the whole document ---	1-23
A	BRUNVAND ET AL.: "CONGESTIVE HEART FAILURE BY VITAMINE D DEFICIENCY" ACTA PAEDIATR., vol. 84, 1995, pages 106-108, XP000867333 cited in the application the whole document ---	1-23

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INTERNATIONAL SEARCH REPORT

Ints. Joint Application No

PCT/EP 99/07720

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOLICK M F: "NONCALCEMIC ACTIONS OF 1,25-DIHYDROXYVITAMIN D3 AND CLINICAL APPLICATIONS" BONE,GB,PERGAMON PRESS., OXFORD, vol. 17, no. 2, 1 August 1995 (1995-08-01), pages 107S-111S, XP002035272 ISSN: 8756-3282 the whole document ---	
A	HAUSSLER ET AL.: "THE VITAMIN D HORMONE AND ITS NUCLEAR RECEPTOR:MOLECULAR ACTIONS AND DISEASE STATES" J ENDOCRINOLOGY, vol. 154, 1997, pages S57-S73, XP000867746 cited in the application the whole document ---	
P,X	VAN SCHOOTEN ET AL.: "PUTATIVE SUSCEPTIBILITY MARKERS OF CORONARY ARTERY DISEASE: ASSOCIATION BETWEEN VDR GENOTYPE, SMOKING, AND AROMATIC DNA ADDUCT LEVELS IN HUMAN RIGHT ATRIAL TISSUE" THE FASEB JOURNAL, vol. 12, October 1998 (1998-10), pages 1409-1417, XP002130406 the whole document -----	1-23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 99/07720

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 16, 17, 19 and 20 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/EP 99/07720

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9740187 A	30-10-1997	AU 2395697 A CA 2251744 A EP 0909330 A US 5939260 A	12-11-1997 30-10-1997 21-04-1999 17-08-1999
WO 9403633 A	17-02-1994	AU 673187 B AU 4690093 A CA 2141300 A EP 0652975 A JP 8501933 T US 5593833 A	31-10-1996 03-03-1994 17-02-1994 17-05-1995 05-03-1996 14-01-1997